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St. John's wort relieves pain in an animal model of migraine

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Abstract

Background: Despite the substantial improvement that antimigraine drugs brought to migraineurs, there is the need for a long-acting and better tolerated migraine treatment than actual pharmacotherapy. St. John's wort (SJW), a medicinal plant endowed with a favourable tolerability profile, showed numerous bioactivities. We here investigated the pain-relieving property of SJW and its main components, hypericin and flavonoids, in a mouse model induced by nitric oxide (NO) donors administration.

Methods: The NO donors nitroglycerin and sodium nitroprusside (SNP) induced allodynia (cold plate test) and hyperalgesia (hot plate test). Western blotting experiments were performed to detect c-Fos and protein kinase C (PKC) expression within periaqueductal grey matter (PAG).

Results: A single oral administration of an SJW dried extract (5 mg/kg p.o.) produced a prolonged relief from pain hypersensitivity. Similarly, preventive SJW administration increased the latency to the induction of hyperalgesia and reduced the duration of the painful symptomatology. Among SJW main components, hypericin showed a similar profile of activity, whereas flavonoids were devoid of any antihyperalgesic effect. To clarify the cellular pathways involved in the SJW mechanism of action, we examined the effects induced by the herbal drug on PKC. NO donors' administration upregulated and increased phosphorylation of PKCγ and PKCε isoforms within PAG that was prevented by SJW treatment. The absence of behavioural side effects or altered animals' locomotor activity by SJW was demonstrated.

Conclusions: These results suggest SJW as a safe therapeutic perspective for migraine pain, and indicate PKC as an innovative target for antimigraine therapy.

1. Introduction

In the past two decades, experimental and clinical research has led to considerable advances in understanding the pathophysiological mechanisms of migraine, and new options for acute and prophylactic treatment have emerged. The discovery of triptans was the most significant step forward in the treatment of acute migraine, but not all migraineurs respond to triptans, and many who do benefit from them have troublesome adverse effects or do not experience sustained pain relief (Ferrari et al., 2001). For this reason, there is still a significant need for well-tolerated new drugs that provide effective, quick and sustained relief from migraine pain (Ferrari et al., 2010; Weatherall et al., 2010; Diener et al., 2011). A number of drugs belonging to various pharmacological classes and deliverable by several routes are now available both for the acute and the preventive treatments of migraine (Magis and Schoenen, 2011; Olesen and Ashina, 2011). Nevertheless, disability and satisfaction remain low in many subjects because treatments are not accessible, not optimized, not effective or simply not tolerated.

What's already known about this topic?
- A number of antimigraine drugs belonging to different pharmacological classes are available, which brought a substantial improvement in the migraineurs' quality of life. However, there is the need for a long-acting and better tolerated migraine treatment than current pharmacotherapy.

What does this study add?
- SJW might represent a long-acting, safe and tolerable perspective for antimigraine therapy.

Hypericum perforatum L., commonly called St. John's wort (SJW), has been used for centuries as medicinal plant. More recently, it has received attention for its efficacy against mild to moderate depression, comparable to that of standard antidepressants, with a favourable incidence of side effects (Kasper et al., 2010). Despite pharmacological studies on SJW that have focused on its antidepressant activity, some studies have documented other bioactivities produced by this herbal plant, such as antibacterial (Saddiqe et al., 2010) and antiviral (Birt et al., 2009) activities. SJW is also endowed with anti-inflammatory activity following not only topic (Sosa et al., 2007), but also systemic administration (Mattace Raso et al., 2002). Recently, the analgesic activity against acute pain (Galeotti et al., 2010a) and the capability to relieve neuropathic pain in different animal models (Galeotti et al., 2010b) were observed after oral administration of SJW.

Based on the data implicating the anti-inflammatory, analgesic and antineuropathic bioactivities of SJW, we investigated the efficacy of a SJW dried extract in an animal model of meningeal nociception induced by administration of nitric oxide (NO) donors to evaluate its potential antimigraine activity. *Hypericum* extract contains at least 10 active constituents that may contribute to its pharmacological effects (Greeson et al., 2001). Among them, phloroglucinols (e.g. hyperforin), naphthodianthrones (e.g. hypericin) and the flavonoids (e.g. hyperoside, quercetin) are the most abundant ones and the main responsible agents for the traditional use of this herbaceous plant. The role of the SJW main components was also investigated in order to elucidate their involvement in the modulation of pain perception. Furthermore, elucidating the cellular mechanism of action of SJW might help clarify the pathophysiology of migraine. We, then, investigated the influence on key molecules involved in pain sensation by SJW and components. In particular, we focused our attention on the modulation of the activity of protein kinase C (PKC), a family of enzymes highly involved in pain perception (Velazquez et al., 2007).

2. Materials and methods

2.1 Animals

Male Swiss albino mice (20–22 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Ten mice were housed per cage (26 × 41 cm). The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12-h light/dark cycle, light on at 7 a.m. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Drug administration

2.2.1 Behavioural studies

The NO donors nitroglycerin (GNT) (10 mg/kg, Bioindustria L.I.M., Italy), dissolved in 10% ethylene glycol in saline (0.9% NaCl), and sodium nitroprusside (SNP) (1 mg/kg, Sigma, Italy), dissolved in saline, were administered intraperitoneally (i.p.) as previously reported (Tassorelli et al., 2003), and the behavioural tests were performed 1–6 h after intraperitoneal administration.

Hypericum perforatum (SJW) dried extract containing 0.32% of total hypericins (Indena Research Laboratories, Settala, Milan, Italy), hypericin, hyperoside, quercetin and amentoflavone (Sigma, Milan, Italy) were dissolved in 1% carboxymethylcellulose solution immediately before use and administered by oral gavage. The doses of hypericin (0.01 mg/kg), quercetin (0.0415 mg/kg), amentoflavone (0.0029 mg/kg) and hyperoside (0.3175 mg/kg) correspond to the amount of each component present in a 5-mg/kg preparation of SJW dried.

To evaluate the capability of SJW to counteract the pain hypersensitivity produced by NO donors, the herbal drug and its main components were administered 150 min after GTN or SNP. Ergotamine tartrate (0.1 mg/kg i.p.) and indomethacin (1 mg/kg i.p.) (Calbiochem, Milan, Italy), used as antimigraine reference drugs, and the PKC blocker calphostin C [0.05–0.2 μg

per mouse intracerebroventricularly (i.c.v.)] (Calbiochem, Milan, Italy) were administered 150 min after NO donors.

To investigate the activity of SJW to prevent NO donor-induced hypersensitivity to pain, SJW, hypericin, ergotamine and indomethacin were administered 30 min before GNT or SNP treatment, whereas calphostin C was administered 5 min before NO donors.

Vehicles used to dissolve drugs were tested for the absence of any effect on pain threshold in comparison with saline-treated and naïve mice.

2.2.2 Western blotting experiments

Experiments were conducted on periaqueductal grey matter (PAG) of naïve and GTN- and SNP-treated mice. Brain areas were removed 1 or 4 h after NO donors' administration. For time course experiments, PAG was removed 1, 2, 4 and 6 h after NO donors' administration.

Doses and administration schedules of compounds used were chosen on the basis of time course and dose-response curves performed in our laboratory.

Intracerebroventricular (i.c.v.) administration was performed under ether anaesthesia, as previously described (Galeotti et al., 2003). Briefly, during anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4-mm external diameter, hypodermic needle attached to a 10-μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl were then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5 μl of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated, and the percentage of correct injections was determined to be 95%. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5 μl per mouse.

2.3 Cold plate

For assessment of cold allodynia, mice were placed on a cold plate that is maintained at a temperature of 4 ± 0.1 °C. Reaction times (s) were measured with a stopwatch before and 1, 2, 4 and 6 h after administration of the NO donors. The time between placements of a mouse on the plate and licking or lifting of a hind paw was measured with a digital timer. An arbitrary cut-off time of 60 s was adopted. Ten to fifteen mice per group were tested.

2.4 Hot plate

Mice were placed inside a stainless steel container, which was set thermostatically at 50.0 ± 0.1 °C in a precision water bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s) were measured with a stopwatch before and 1, 2, 4 and 6 h after administration of the NO donors. The endpoint used was the licking of the fore or hind paws. An arbitrary cut-off time of 60 s was adopted. Ten to fifteen mice per group were tested.

2.5 Motor coordination

The motor coordination was assessed by using the rotarod test. The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. Those mice scoring less than three and more than six falls in the pretest were rejected (20%). The number of falls was measured before (pretest) and 1, 2, 4 and 6 h after the administration of the NO donors. Ten mice per group were used.

2.6 Locomotor activity

The locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40-cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed at the centre of the board one by one and allowed to move about freely for a period of 5 min each. Two photobeams, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. Experiments were performed 4 h after administration of the NO donors. Ten mice per group were tested.

2.7 Western blot analysis

Samples to conduct Western blotting experiments were collected 1 or 4 h after the GTN (10 mg/kg i.p.) or SNP (1 mg/kg i.p.) treatment. PAG was homogenized in an homogenization buffer containing 25 mM Tris-HCl pH = 7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPP, 4 mM PNFF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9000 xg for 15 min at 4 °C, the low speed pellet was discarded. Protein concentration was quantified using Bradford's method (protein assay kit, Bio-Rad Laboratories, Milan, Italy). Membrane homogenates (10 μg) made from PAG of GTN-, SNP-treated and naïve mice were separated on 10% SDS-PAGE, and transferred onto nitrocellulose membranes (90 min at 120 V) using standard procedures. Membrane were blocked in PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 120 min. Following washings, blots were incubated overnight at 4 °C with specific antibodies against PKCγ phosphorylated on Thr514 (pPKCγ, 1:1000 dilution); c-Fos (1:1000) (Biosource, Camarillo, CA, USA); PKCε phosphorylated on Ser729 (pPKCε, 1:750); PKCγ (1:1000); and PKCε (1:800) (Santa Cruz Biotechnology Inc, CA, USA). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10,000) and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy). Exposition and developing time used was standardized for all the blots. Optical density measurements were performed by dividing the intensity of the bands by the intensity of the housekeeping protein β-actin, used as loading control, at each time point. Measurements in control samples were assigned a relative value of 100%.

2.8 Statistical analysis

All experimental results were given as the mean ± standard deviation. Analysis of variance followed by Tukey post hoc test was used for statistical analysis.

3. Results

3.1 Thermal pain hypersensitivity induced by NO donors' administration

To investigate the property of SJW that relieves migraine pain, we used an animal model obtained by administration of the NO donors SNP and nitroglycerin (GTN) in mice. Administration of SNP produced allodynia as revealed by the cold plate test (Fig. 1A). The reaction times to the cold stimulus remained unmodified following SNP 0.5 mg/kg i.p. Animals treated with SNP 1 and 2 mg/kg i.p. showed reduced reaction times between 1 and 4 h after NO donor administration, and returned to control values 6 h after SNP injection. Similarly, the administration of GTN (10 mg/kg i.p.) produced cold allodynia with a profile similar to that produced by SNP (Fig. 1B). Following NO donors' treatment, a thermal hyperalgesia was observed in the hot plate test with a similar time course to the cold allodynia. Mice showed reduced licking latency values 2 and 4 h after SNP (1 mg/kg; Fig. 1C) and GTN administration (10 mg/kg; Fig 1D). GTN and SNP cold allodynia and heat hyperalgesia peaked after 4 h, consistent with a typical migraine attack in migraineurs that develops 4–6 h after GTN infusion (Olesen et al., 1993).

c-Fos, the protein product of the immediate early gene *c-fos*, has been widely used as a marker of neuronal activation, and particularly, as a marker of pain (Harris, 1998). We, therefore, detected the c-Fos protein content in a brain area highly related to pain perception. A rapid and progressive increase of c-Fos expression within the PAG was observed after SNP (1 mg/kg; Fig. 1E) and GTN administration (10 mg/kg; Fig. 1F). The c-Fos upregulation followed the same time course of the pain hypersensitivity observed in cold and hot plate tests (Fig. 1A–D), showing a time course consistent with migraine attacks in susceptible individuals.

3.2 Effect of NO donors and SJW on mouse locomotor activity

The NO donor-induced hypersensitivity to pain was not accompanied by the induction of side effects. The spontaneous mobility and exploratory activity of mice treated with SNP (Fig. 1G) or GTN (data not shown) were unmodified in comparison with the control group. In the same experimental conditions, co-administration with SJW as pretreatment (30 min before) or treatment 150 min after NO donors did not modify both parameters evaluated (Fig. 1G). NO donors did not alter locomotor activity of treated animals at any time point, as indicated by the rotarod test results. Co-administration with SJW never modified the number of falls from the rotating rod (Fig. 1H).

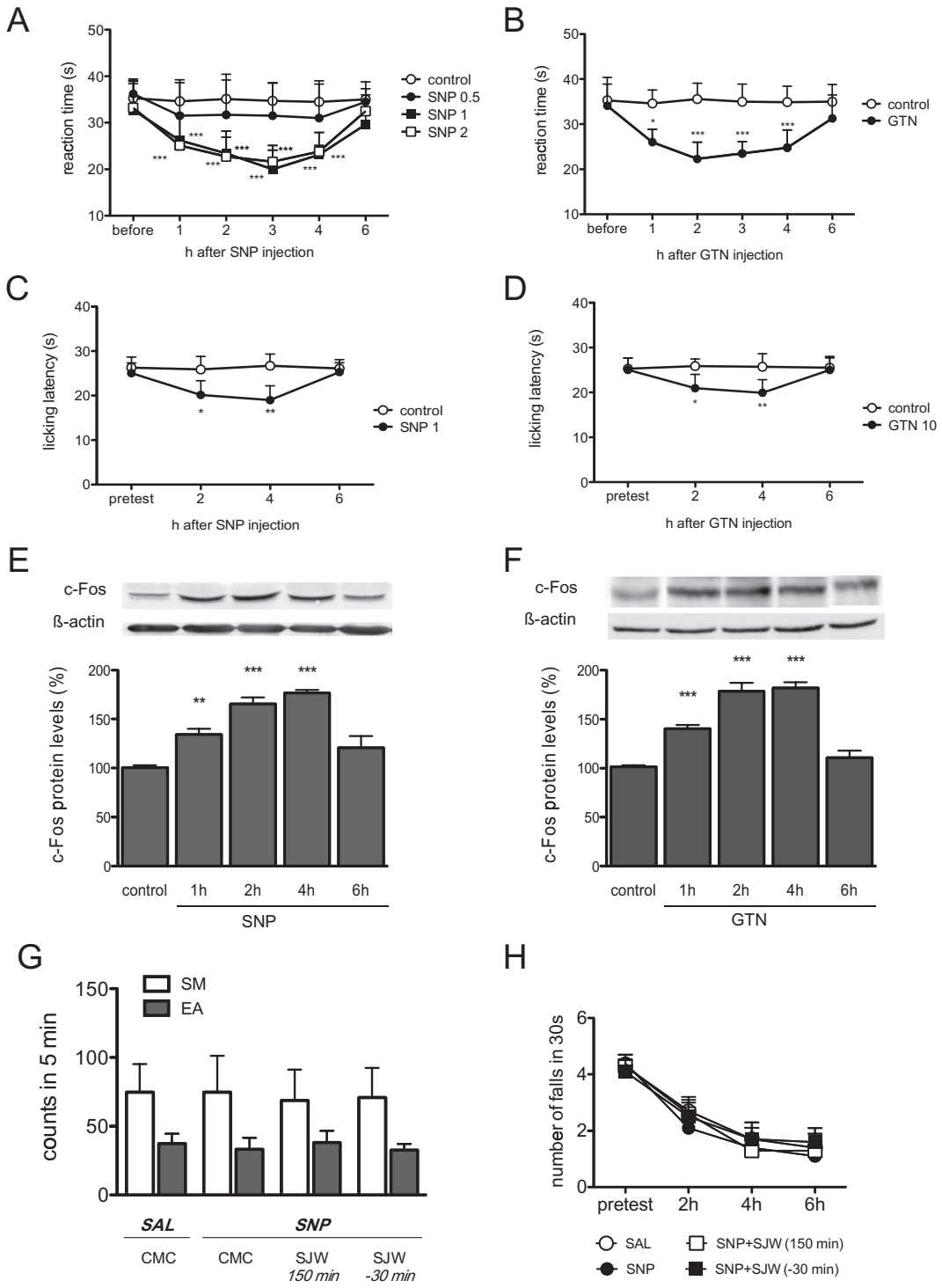

Figure 1 NO donors decrease the pain threshold. Administration of sodium nitroprusside (SNP) at the doses of 0.5, 1, 2 mg/kg i.p. (A), or nitroglycerin (GTN) at the dose of 10 mg/kg i.p., induces cold allodynia, evaluated in the cold plate test (B). A thermal hyperalgesia, evaluated in the hot plate test, was also detected 2 and 4 h after SNP (1 mg/kg) (C) or GTN (10 mg/kg) injection in comparison with the corresponding vehicle-treated group, used as control (D). NO donors modulate c-Fos expression in the PAG following SNP (E) or GTN (F) administration with a similar time course. The columns represent the densitometric quantitation of immunoreactive protein expressed relative to control. Representative immunoblots are reported at the top of each panel. SNP did not alter spontaneous mobility, inspection activity (hole-board test) (G) or motor coordination (rotarod test) (H) at any time point. Co-administration with SJW did not induce any alteration of the parameters evaluated. *$p < 0.05$, *$p < 0.01$, ***$p < 0.001$ compared with control group.

3.3 SJW reversed pain hypersensitivity induced by NO donors' administration

Oral administration of a SJW dried extract 1 mg/kg p.o. was unable to modify pain hypersensitivity observed in the cold plate test following SNP administration (Fig. 2A). At 5 mg/kg p.o., SJW completely reversed the cold allodynia as shown by the reaction times similar to control values (Fig. 2A). To elucidate the mechanism of the antimigraine activity of SJW, we investigated the effects produced by some of the main components of this herbal drug administered in a concentration corresponding to the content present in a 5 mg/kg preparation of SJW. The administration of hypericin (hyp; 0.01 mg/kg p.o.) reversed the cold allodynia with a similar profile to SJW (Fig. 2B). Conversely, oral administration of the flavonoids quercetin (0.0415 mg/kg), amentoflavone (0.0029 mg/kg) or hyperoside (0.3175) was devoid of any effect (Fig. 2C).

SJW (5 mg/kg p.o.) was able to reverse pain hypersensitivity induced by GNT as well (Fig. 2D). Similar results were obtained after oral administration of hypericin (Fig. 2E), whereas quercetin (Fig. 2F), amentoflavone and hyperoside (data not shown) were unable to relieve from cold allodynia.

The administration of SWJ reversed the thermal hyperalgesia produced by NO donors in the hot plate test. The administration of GTN reduced the pain threshold 2 and 4 h after administration. Oral administration of SJW counteracted this effect, showing licking latencies values comparable to control group (Fig. 2G). Similar results were obtained with hypericin (Fig. 2G) and quercetin (Fig. 2H), whereas amentoflavone and hyperoside were devoid of any effect (Fig. 2H). The same results were obtained against the hyperalgesia induced by SNP (Fig. 2I).

3.4 SJW antimigraine activity requires a PKC blockade

To confirm that the antimigraine effect of SJW was related to the PKC-blocking properties of hypericin, we detected the effect produced by the PKC blocker calphostin C on cold allodynia. The administration of calphostin C reversed the pain hypersensitivity induced by SNP with a dose-dependent duration of effect. At 3 h, the dose of 0.05 μg slightly increased pain threshold without reaching the statistical significance, at 0.1 μg counteracted the NO donors' pain hypersensitivity and at 0.2 μg the effect was more prolonged being significant up to 4 h (Fig. 3A). Calphostin C reversed the cold allodynia induced by GTN as well (Fig. 3B). We also conduced Western blotting experiments in the PAG area to detect the expression of the PKC isoforms mainly involved in pain modulation. Time course experiments showed that the expression of the PKCγ and PKCε isoforms was increased following NO donors' administration, with a peak at 2–4 h after treatment. A robust increase of the phosphorylation of both PKC isoforms was also detected between 1 and 4 h after GTN or SNP administration (see Fig. S1). Oral administration of SJW or hypericin completely reversed the pPKCγ (Fig. 3C) and pPKCε upregulation (Fig. 3D) detected 4 h after the administration of SNP or GTN in coincidence with their counteracting effect on pain hypersensitivity evaluated in the cold plate test. Since calphostin C is a PKC inhibitor not-isoform specific, to validate our results we detected the effect produced by calphostin C on PKCγ and PKCε phosphorylation. We observed a complete prevention of pPKCγ and pPKCε upregulation by i.c.v. administration of the effective dose of calphostin C 4 h after NO donors' treatment (see Fig. S1).

3.5 Prevention of painful symptomatology by SJW

SJW was investigated as preventive pharmacological treatment for painful symptomatology. Animals received an oral administration of SJW 30 min before injection of NO donors. Treatment was unable to completely prevent pain hypersensitivity, but the latency to the induction of pain was increased and the duration of the painful period was shortened. The absence of any hypersensitivity to pain was observed up to 2 h after SNP (Fig. 4A) or GTN (data not shown) treatment. Starting from 3 h after administration, NO donor-induced allodynia was detectable also in the SJW-treated group. Oral administration of purified hypericin prevented cold allodynia, but starting from 2 h after NO donor treatment, the antiallodynic effect disappeared (Fig. 4B). The administration of calphostin C 5 min before NO donors prevented cold allodynia up to 1 h towards SNP- (Fig. 4C) or GTN-induced (Fig. 4D) hypersensitivity to pain, showing a time course similar to hypericin.

Western blotting experiments showed that the SJW and hypericin preventive effect was related to the capability to prevent PKC hyperphosphorylation in the PAG. This activity as detected 1 h after SNP or GNT administration, in coincidence with the SJW peak of preventive activity. Following NO donors' treatment, an increase of pPKCγ levels was observed. A pretreatment with SJW or hypericin completely prevented PKCγ hyperphosphorylation (Fig. 4E). SNP and GTN

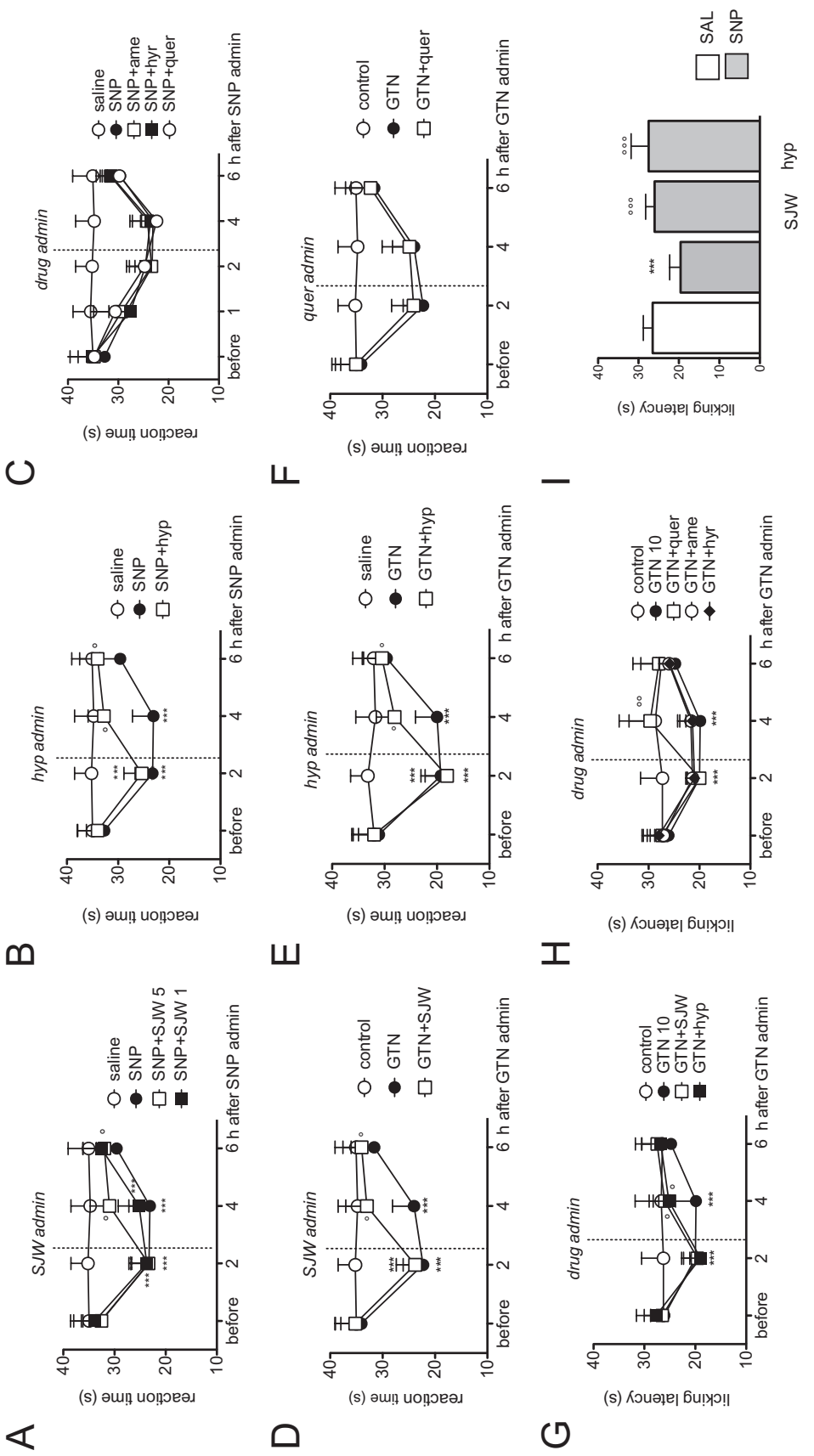

Figure 2 Effect of SJW and components on NO donor-induced allodynia and heat hyperalgesia. (A) SJW counteracted SNP-induced cold allodynia at 5 mg/kg p.o. Among the SJW main components, hypericin 0.01 mg/kg p.o. (hyp) (B) showed an efficacy profile similar to SJW, whereas quercetin 0.0415 mg/kg p.o. (quer), amentoflavone 0.0029 mg/kg p.o. (ame) and hyperoside 0.3175 mg/kg p.o. (hyr) were ineffective (C). SJW 5 mg/kg p.o. (D) and purified hypericin 0.01 mg/kg p.o. (hyp) (E) showed antiallodynic effect also towards GTN-induced cold allodynia in the mouse cold plate test, whereas quercetin 0.0415 mg/kg p.o. (quer) was ineffective (F). (G) GTN reduced pain threshold 2 and 4 h after administration in the hot plate test. SJW (5 mg/kg p.o.) and hypericin (hyp; 0.01 mg/kg p.o.) showed antihyperalgesic properties. (H) Quercetin (quer; 0.0415 mg/kg p.o.) counteracted hyperalgesia, whereas amentoflavone (ame; 0.0029 mg/kg p.o.) and hyperoside (hyr; 0.3175 mg/kg p.o.) were ineffective. (I) SWJ and hypericin also prevented SNP-induced thermal hyperalgesia. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ compared with NO donor-treated group at 2 h.

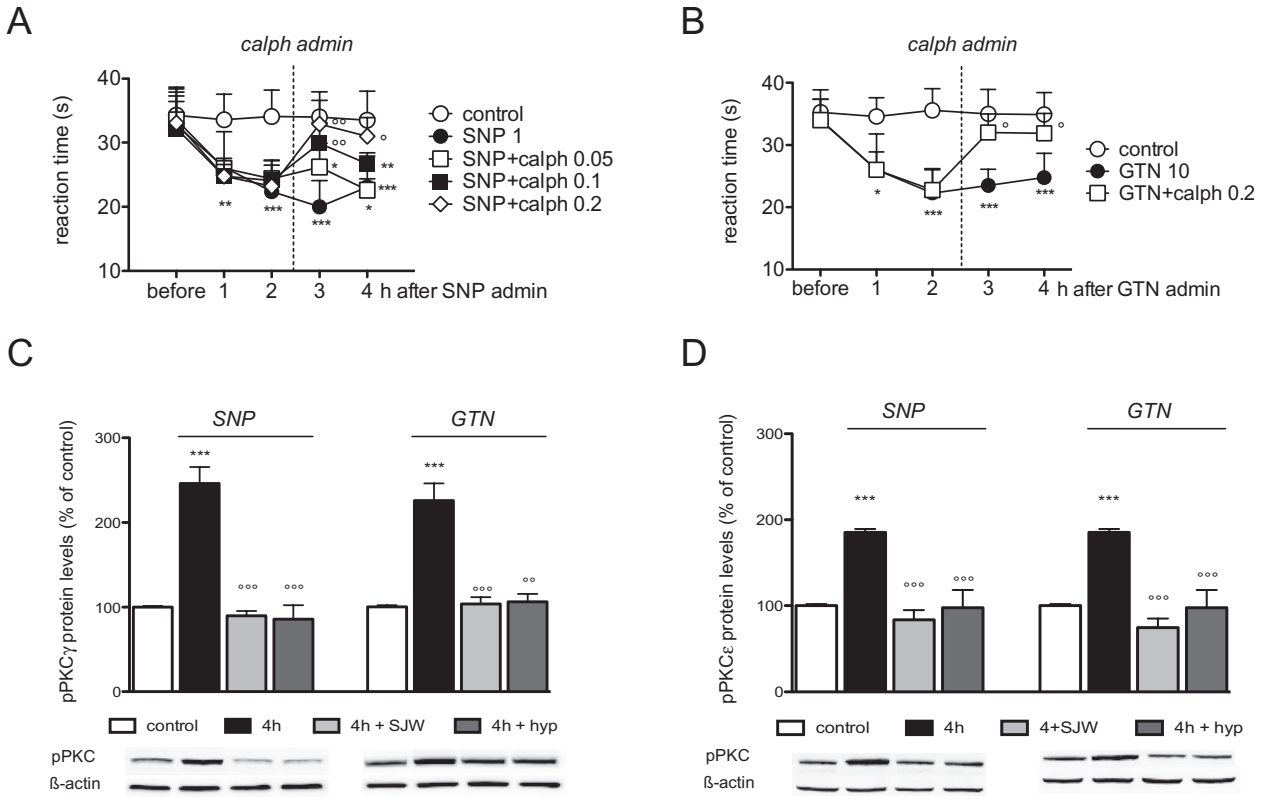

Figure 3 Modulation of PKC phosphorylation by SJW and hypericin. The PKC blocker calphostin C (0.05–0.2 μg) reversed cold allodynia induced by SNP (A), or GTN (B) when i.c.v. administered 150 min after the NO donor. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with control group. $°p < 0.05$, $°°p < 0.01$; $°°°p < 0.001$ compared with corresponding NO donor-treated group at 2 h. SNP and GTN increased phosphorylation of PKCγ (C) and PKCε (D) within the periaqueductal grey matter (PAG) 4 h after administration that was completely prevented by oral administration of SJW and hypericin. The columns represent the densitometric quantitation of immunoreactive protein expressed relative to control. Representative immunoblots are reported in each panel. $***p < 0.001$ compared with control group. $°°p < 0.01$, $°°°p < 0.001$ compared with corresponding NO donor-treated group.

also increased pPKCε levels 1 h after administration, effect completely prevented by pretreatment with SJW or hypericin (Fig. 4F).

3.6 Effect of ergotamine and indomethacin on NO donor-induced pain hypersensitivity

The antiallodynic effect produced by SJW and hypericin was comparable to that exerted by ergotamine (0.1 mg/kg i.p.) and indomethacin (1 mg/kg i.p.), used as antimigraine reference drugs, towards GTN- (Fig. 5A) and SNP-induced (Fig. 5B) cold allodynia. The antihyperalgesic effect produced by ergotamine and indomethacin was of the same intensity to that exerted by SJW.

Similarly, both reference drugs counteracted heat hyperalgesia 3 and 4 h after GTN (Fig. 5C) or SNP (Fig. 5D) administration, showing the same activity profile observed with SJW.

Also, the preventive activity of SJW was similar to that produced by ergotamine and indomethacin towards GTN- (Fig. 5E) and SNP-induced (Fig. 5F) cold allodynia. Both reference drugs prevented the hypersensitivity to pain at 1 h after NO donors' administration, whereas starting from 2 h, the NO donor-induced allodynia was again detectable (Fig. 5E,F).

4. Discussion

Our study showed that a single oral administration of a SJW dried extract prevented pain hypersensitivity and neuronal activation in a mouse model of meningeal nociception obtained by the systemic administration of the GTN and SNP.

A common clinical feature of an untreated migraine attack is hyperalgesia, and allodynia affecting the scalp, face, and contiguous regions of the neck and

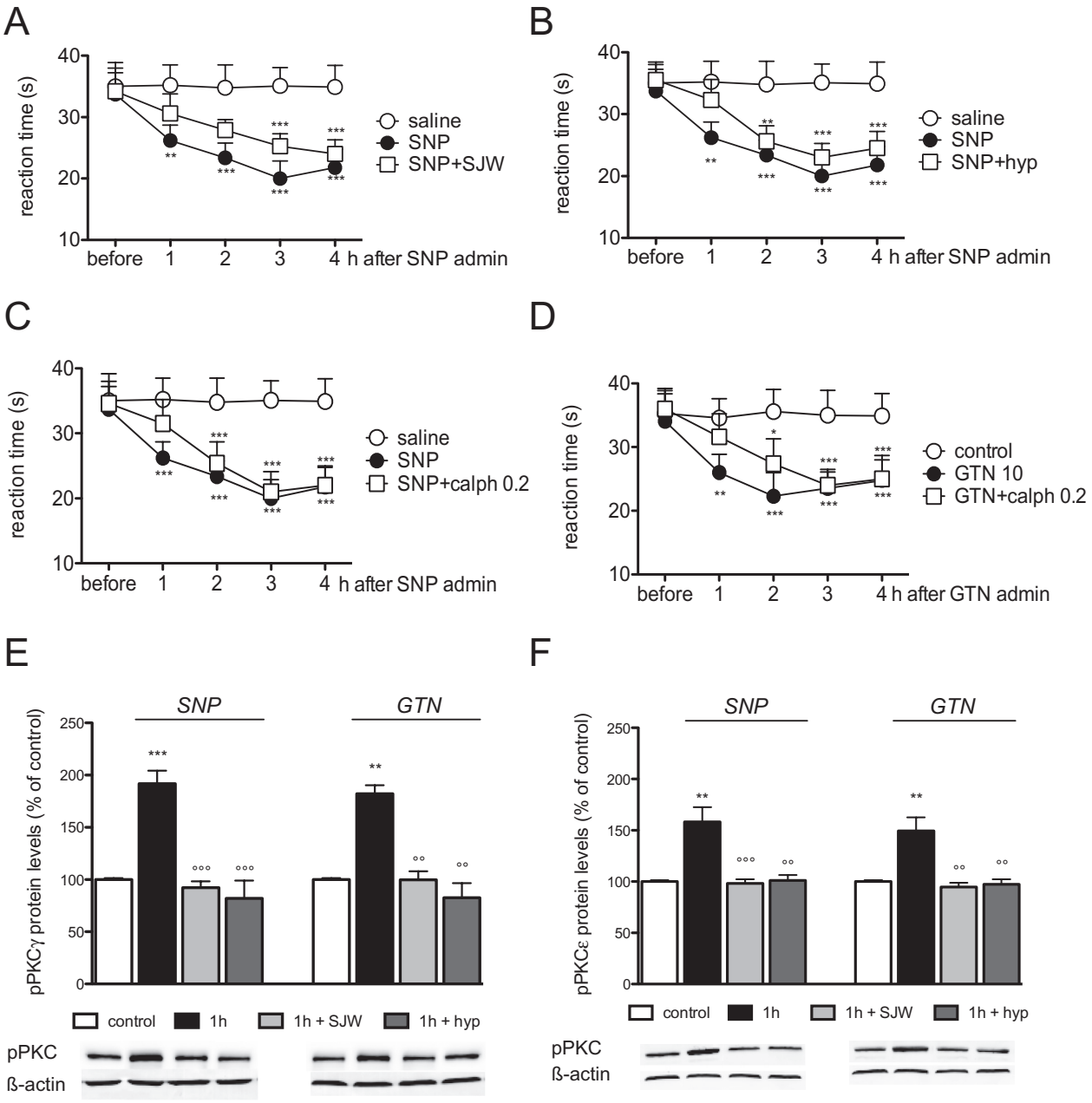

Figure 4 Oral administration of SJW and hypericin prevented NO donor-induced hypersensitivity to pain through a PKC-dependent mechanism. Pretreatment with SJW 5 mg/kg p.o. (A) and hypericin 0.01 mg/kg p.o. (B), administered 30 min before SNP, prevented cold allodynia induced by the NO donor. Calphostin C, administered i.c.v. 5 min before NO donors, prevented the hypersensitivity to pain induced by SNP (C) and GTN (D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control group. SNP and GTN upregulated pPKCγ (E) and pPKCε (F) expression within the periaqueductal grey matter (PAG) 1 h after administration. Oral pretreatment with SJW and hypericin completely prevented the PKC hyperphosphorylation. The columns represent the densitometric quantitation of immunoreactive protein expressed relative to control. Representative immunoblots are reported in each panel. **$p < 0.01$, ***$p < 0.001$ compared with control group. °°$p < 0.01$, °°°$p < 0.001$ compared with corresponding NO donor-treated group.

torso (Burstein et al., 2000). A single oral administration of a 5-mg/kg SJW dried extract reversed the allodynia and hyperalgesia induced by NO donors, producing a prolonged increase of pain threshold. Allodynia has been recognized in migraine since the 19th century, with clinic- (Selby and Lance, 1960) and population- (Bigal et al., 2008) based studies showing that it is seen in about 75% of migraine attacks (Burstein et al., 2000). Allodynia is a clinical reflection of sensitization, and both central and peripheral sensiti-

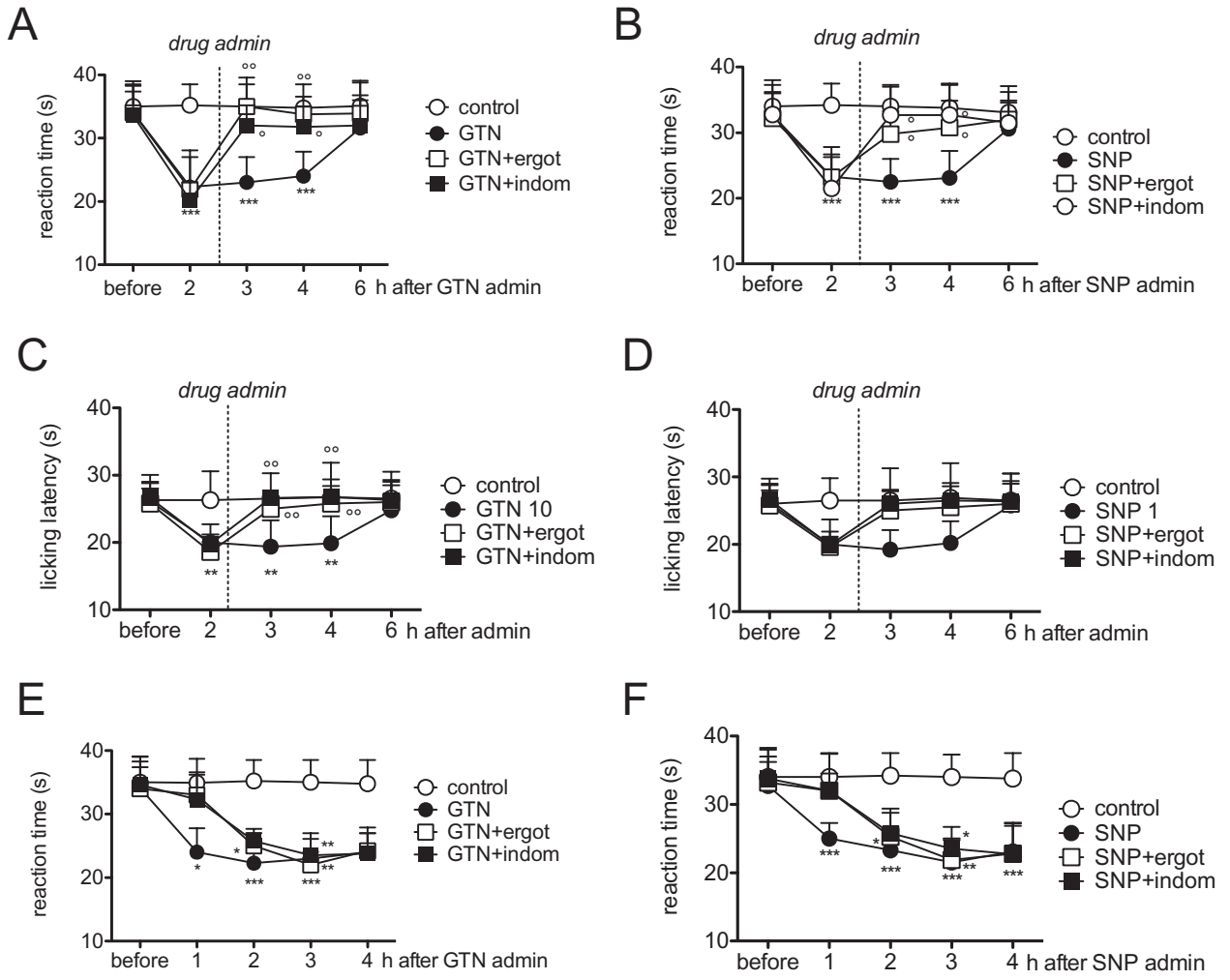

Figure 5 Ergotamine and indomethacin reversal of pain hypersensitivity induced by NO donors. (A) GNT (10 mg/kg i.p.) induced cold allodynia in mice that was reversed by ergotamine (0.1 mg/kg i.p.) and indomethacin (1 mg/kg i.p.), administered 150 min after GTN. (B) A single administration of SNP (1 mg/kg ip.) showed a marked reduction of reaction times in the cold plate test that was reversed by ergotamine (0.1 mg/kg i.p.) and indomethacin (1 mg/kg i.p.), administered 150 min after SNP. GTN (C) and SNP (D) induced a thermal hyperalgesia in the mouse hot plate test that was reversed by ergotamine (0.1 mg/kg i.p.) and indomethacin (1 mg/kg i.p.), administered 150 min after NO donors. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with control group. $°p < 0.05$, $°°p < 0.01$; $°°°p < 0.001$ compared with corresponding NO donor-treated group at 2 h. Ergotamine and indomethacin prevented pain hypersensitivity induced by GNT (10 mg/kg i.p.) (E) or SNP (F) when administered 30 min before NO donors.

zations are important insofar as they both influence attacks and perhaps disease progression (Burstein, 2001; Cooke et al., 2007). Allodynia is not only a clinical marker for sensitization of central pain pathways since differences in treatment efficacy during migraine attacks have been demonstrated based on the presence or absence of allodynia (Burstein et al., 2004), showing allodynia as an important marker for treatment efficacy. The antiallodynic activity, showed simultaneously to the antihyperalgesic activity, by SJW appears to be of particular relevance for the treatment of migraine pain.

SJW dried extract contains numerous active components (Greeson et al., 2001). The effects produced by the main constituents were investigated in order to identify the component responsible for the relief from pain and allodynia, and then to better elucidate its mechanism of action. The naphtodiantrone hypericin reversed pain hypersensitivity with a similar efficacy and time course of SJW. Hypericin has long been known to be related to pharmacological actions of SJW. The antidepressive, antineoplastic, antitumor and antiviral activities of hypericin have been reported (Kubin et al., 2005), and recently its analgesic and

antineuropathic properties have been demonstrated (Galeotti et al., 2010a,b). We here report the antiallodynic and antihyperalgesic activity of this SJW component, suggesting it as a main constituent of the herbal drug in the control of migraine pain.

To clarify the cellular mechanism of the antimigraine activity of SJW, we focused our attention on the molecular pathways modulated by hypericin. Enzyme assays performed on rat brain demonstrated that hypericin is a potent and selective inhibitor of the PKC (Takahashi et al., 1989), an enzyme highly involved in pain modulation (Velazquez et al., 2007). PKC is a family of serine/threonine kinases that are divided into three groups based on calcium and diacylglycerol dependence: conventional (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical (ζ, λ/ι) (Way et al., 2000). We examined the involvement of PKCε and PKCγ since they appear to be the isoforms with a prominent role in the modulation of pain perception (Velazquez et al., 2007). We detected a specific upregulation and increased phosphorylation of PKCε and PKCγ isoforms within PAG of GTN- and SNP treated mice, and their modulation temporally coincided with the presence of allodynia and hyperalgesia. Oral administration of SJW and hypericin prevented the NO donor-induced hyperphosphorylation of both PKC isoforms. The blockade of PKC activity appears a fundamental step in the mechanism of action of SJW since also its analgesic and antineuropathic activity is related to the prevention of cerebral PKC phosphorylation (Galeotti et al., 2010a,b).

A role of flavonoids in the SJW-induced modulation of pain sensation cannot be excluded since it has been reported that flavonoids contribute to the anti-inflammatory properties of the plant (Tedeschi et al., 2003), and an antinociceptive activity has been demonstrated for the flavonoid myricitrin (Meotti et al., 2006). Hyperoside, the most abundant flavonoid present in the SJW dried extract, showed neither antiallodynic nor antihyperalgesic activity. Anti-inflammatory and analgesic properties were reported for the flavonoid amentoflavone (Kim et al., 2008), but when administered in mice at a concentration corresponding to the amount present in a 5-mg/kg preparation of SJW, it was devoid of any effect. Quercetin was unable to reverse allodynia even if it resulted to be able to relieve from heat hyperalgesia. This is not surprising since quercetin, similarly to hypericin, is endowed with PKC-blocking properties. A quercetin potency about 30 times lower (Ferriola et al., 1989) than hypericin (Takahashi et al., 1989) might explain the lower efficacy observed.

These results have highlighted the involvement of a PKC-mediated pathway in the mechanism of antimigraine action of SJW that might reflect the presence of a PKC hyperphosphorylation during a migraine attack related to the induction of both allodynia and hyperalgesia. This hypothesis is supported by clinical evidence illustrating that tamoxifen, the only agent with documented and appreciable central PKC-inhibitory activity approved for human use (Baltuch et al., 1993), has shown promise in treating migraine as attested by case reports (Powles, 1986; Smitherman and Kolivas, 2009) and clinical studies (O'Dea and Davis, 1990; Cuzick et al., 2007). On the light of these promising results, we can suggest PKC as an innovative target for migraine pain.

SJW and hypericin, when administered before NO donors injection, prolonged the latency to the induction of pain hypersensitivity and reduced the duration of the painful symptomatology. These results appear of particular relevance, suggesting SJW as a compound that is able not only to abort a painful condition, but also to partially prevent it. All migraine patients need acute treatment for each attack, but those with frequent attacks need also a prophylactic pharmacotherapy. Given that almost all current models of migraine are acute, the number of emerging treatments for acute attacks is by far much higher than the number for prophylaxis.

The antiallodynic and antihyperalgesic effect produced by SJW is comparable to that produced by ergotamine and indomethacin, used as reference antimigraine drugs. Furthermore, the dose of SJW used (0.016 mg of total hypericins) was largely lower than those required to induce antidepressant (1.8–2.7 mg/die of total hypericins) (Kasper et al., 2010), analgesic and antineuropathic activities (0.96 mg of total hypericins) (Galeotti et al., 2010a,b). We can suppose that SJW induced a selective antimigraine effect in this animal model that is not secondary to its antidepressant or analgesic property. It has been demonstrated that the SJW bioactivities are endowed with a bell-shaped trend (Galeotti et al., 2010a,b). This implies that the dose of SJW to be administered should be carefully chosen on the basis of the pharmacological effect to be obtained.

An important drawback of the drugs used as antimigraine treatment is the high occurrence of side effects. Conversely, SJW is endowed with a favourable tolerability and safety profile (Rahimi et al., 2009). We further demonstrated the tolerability of SJW at doses that are able to prevent pain hypersensitivity in the migraine model. This herbal drug neither produced detectable modification of animals' gross behaviour,

nor altered locomotor activity. Recently, interactions of SJW with prescription drugs have been reported. SJW, at the dose recommended for the treatment of mild to moderate depression, is a potent inducer of cytochrome P450 enzymes, resulting in decrease plasma concentration of a number of drugs used in co-medication (i.e. digoxin, warfarin, oral contraceptives, etc.) (Whitten et al., 2006). Recent studies could show that the degree of enzyme induction by SJW correlates strongly with the amount of hyperforin found in the product (Madabushi et al., 2006). We observed that SJW counteracts pain hypersensitivity at very low doses containing an amount of hyperforin unable to produce clinical significant interactions (Madabushi et al., 2006; Whitten et al., 2006).

Taken together, these data support the conclusion that SJW prevent allodynia and hyperalgesia produced by NO donors' administration. These effects are secondary to the presence of hypericin that acts through a specific inhibition of PKC activation. We can suppose that SJW represents an important and safe therapeutic perspective for the treatment of migraine attacks.

Author contributions

All authors discussed the results and commented on the manuscript.

References

Baltuch, G.H., Couldwell, W.T., Villemure, J.G., Yong, V.W. (1993). Protein kinase C inhibitors suppress cell growth in established and low-passage glioma cell lines: A comparison between staurosporine and tamoxifen. *Neurosurgery* 33, 495–501.

Bigal, M.E., Ashina, S., Burstein, R., Reed, M.L., Buse, D., Serrano, D., Lipton, R.B., AMPP Group. (2008). Prevalence and characteristics of allodynia in headache sufferers: A population study. *Neurology* 70, 1525–1533.

Birt, D.F., Widrlechner, M.P., Hammer, K.D.P., Hilliwig, M.L., Wei, J., Kraus, G.A., Murphy, P.A., McCoy, J., Wurtele, E.S., Neighbors, J.D., Wiemer, D.F., Maury, W.J., Price, J.P. (2009). *Hypericum* in infection: Identification of anti-viral and anti-inflammatory constituents. *Pharm Biol* 47, 774–782.

Burstein, R. (2001). Deconstructing migraine headache into peripheral and central sensitization. *Pain* 89, 107–110.

Burstein, R., Collins, B., Jakubowski, M. (2004). Defeating migraine pain with triptans: A race against the development of cutaneous allodynia. *Ann Neurol* 55, 19–26.

Burstein, R., Cutrer, F.M., Yarnitsky, D. (2000). The development of cutaneous allodynia during a migraine attack: Clinical evidence for sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. *Brain* 123, 1703–1709.

Cooke, L., Eliasziw, M., Becker, W.J. (2007). Cutaneous allodynia in transformed migraine patients. *Headache* 47, 531–539.

Cuzick, J., Forbes, J.K., Sestak, I., Cawthorn, S., Hamed, H., Holli, K., Howell, A., International Breast cancer Intervention Study I Investigators. (2007). Long-term results of tamoxifen prophylaxis for breast cancer – 96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst* 99, 272–282.

Diener, H.C., Barbanti, P., Dahlof, C., Reuter, U., Habeck, J., Podhorna, J. (2011). BI 44370 TA, an oral CGRP antagonist for the treatment of acute migraine attacks: Results from a phase II study. *Cephalalgia* 31(5), 573–584.

Ferrari, M.D., Farkkila, M., Reuter, U., Pilgrim, A., Davis, C., Krauss, M., Diener, H.C., European COL-144 Investigators. (2010). Acute treatment of migraine with the selective 5-HT1F receptor agonist lasmiditan – a randomised proof of concept trial. *Cephalalgia* 30(10), 1170–1178.

Ferrari, M.D., Roon, K.I., Lipton, R.B., Goadsby, P.J. (2001). Oral triptans (serotonin 5-HT(1B/1D) agonists) in acute migraine treatment: A meta-analysis of 53 trials. *Lancet* 358(9294), 1668–1675.

Ferriola, P.C., Kody, V., Middleton, E. Jr. (1989). Protein kinase C inhibition by plant flavonoids. *Biochem Pharmacol* 38, 1617–1624.

Galeotti, N., Bartolini, A., Ghelardini, C. (2003). The phospholipase C-IP_3 pathway is involved in muscarinic antinociception. *Neuropsychopharmacology* 28, 888–897.

Galeotti, N., Vivoli, E., Bilia, A.R., Bergonzi, M.C., Bartolini, A., Ghelardini, C. (2010a). A prolonged protein kinase C-mediated, opioid-related antinociceptive effect of St John's wort in mice. *J Pain* 11, 149–159.

Galeotti, N., Vivoli, E., Bilia, A.R., Vincieri, F.F., Bartolini, A., Ghelardini, C. (2010b). St John's wort relieves neuropathic pain through a hypericin-mediated inhibition of the protein kinase C γ and ε activity. *Biochem Pharmacol* 79, 1327–1336.

Greeson, J.M., Sanford, B., Monti, D.A. (2001). St. John's wort (*Hypericum perforatum*): A review of the current pharmacological, toxicological, and clinical literature. *Psychopharmacology* 153, 402–414.

Harris, J.A. (1998). Using *c-fos* as marker of pain. *Brain Res Bull* 45, 1–8.

Kasper, S., Caraci, F., Forti, B., Drago, F., Aguglia, E. (2010). Efficacy and tolerability of Hypericum extract for the treatment of mild to moderate depression. *Eur Neuropsychopharmacol* 20, 747–765.

Kim, H.P., Park, H., Son, K.H., Chang, H.W., Kang, S.S. (2008). Biochemical pharmacology of biflavonoids: Implications for anti-inflammatory action. *Arch Pharm Res* 31, 265–273.

Kubin, A., Wierrani, F., Burner, U., Alth, G., Grunberger, W. (2005). Hypericin – The facts about a controversial agent. *Curr Pharm Des* 11, 233–253.

Madabushi, R., Frank, B., Drewelow, B., Derendorf, H., Butterweck, V. (2006). Hyperforin in St. John's wort interactions. *Eur J Clin Pharmacol* 62, 225–233.

Magis, D., Schoenen, J. (2011). Treatment of migraine: Update on new therapies. *Curr Opin Neurol* 24, 203–210.

Mattace Raso, G., Pacilio, M., Di Carlo, G., Esposito, E., Pinto, L., Meli, R. (2002). In-vivo and in-vitro anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perforatum*. *J Pharm Pharmacol* 54, 1379–1383.

Meotti, F.C., Luiz, A.P., Pizzolatti, M.G., Kassuya, C.A.L., Calixto, J.B., Santos, A.R. (2006). Analysis of the antinociceptive effect of the flavonoid myricitrin: Evidence for a role of the L-arginine nitric-oxide and protein kinase C pathways. *J Pharmacol Exp Ther* 316, 789–796.

O'Dea, J.P., Davis, E.H. (1990). Tamoxifen in the treatment of menstrual migraine. *Neurology* 40, 1470–1471.

Olesen, J., Ashina, M. (2011). Emerging migraine treatments and drug targets. *Trends Pharmacol Sci* 32, 352–358.
Olesen, J., Iversen, H.K., Thomsen, L.L. (1993). Nitric oxide supersensitivity: A possible molecular mechanism of migraine pain. *Neuroreport* 4, 1027–1030.
Powles, T.J. (1986). Prevention of migrainous headaches by tamoxifen. *Lancet* 2, 1344.
Rahimi, R., Nikfar, S., Abdollahi, M. (2009). Efficacy and tolerability of *Hypericum perforatum* in major depressive disorder in comparison with selective serotonin reuptake inhibitors: A meta-analysis. *Prog Neuropsychopharmacol Biol Psychiatry* 33, 118–127.
Saddiqe, Z., Naeem, I., Maimoona, A. (2010). A review of the antibacterial activity of *Hypericum perforatum* L. *J Ethnopharmacol* 131, 511–521.
Selby, G., Lance, J.W. (1960). Observations on 500 cases of migraine and allied vascular headache. *J Neurol Neurosurg Psychiatry* 23, 23–32.
Smitherman, T.A., Kolivas, E.D. (2009). Resolution of menstrually related migraine following aggressive treatment for breast cancer. *Headache* 50, 485–496.
Sosa, S., Pace, R., Bornancin, A., Morazzoni, P., Riva, A., Tubaro, A., Della Loggia, R. (2007). Topical anti-inflammatory activity of extracts and compounds from *Hypericum perforatum* L. *J Pharm Pharmacol* 59, 703–709.
Takahashi, I., Nakanishi, S., Kobayashi, E., Nakano, H., Suzuki, K., Tamaoki, T. (1989). Hypericin and pseudohypericin specifically inhibit protein kinase C: Possible relation to their antiretroviral activity. *Biochem Biophys Res Commun* 165, 1207–1212.
Tassorelli, C., Greco, R., Wang, D., Sandrini, M., Sandrini, G., Nappi, G. (2003). Nitroglycerin induces hyperalgesia in rats – A time course study. *Eur J Pharmacol* 464, 159–162.
Tedeschi, E., Menegazi, M., Margotto, D., Suzuki, H., Forstermann, U., Kleinert, H. (2003). Anti-inflammatory actions of St. John's wort: Inhibition of human inducible nitric-oxide synthase expression by down-regulating signal transducer and activator of transcription-1alpha (STAT-1 alpha) activation. *J Pharmacol Exp Ther* 307, 254–261.
Velazquez, K.T., Mohammad, H., Sweitzer, S.M. (2007). Protein kinase C in pain: Involvement of multiple isoforms. *Pharmacol Res* 55, 578–689.
Way, K.J., Chou, E., King, G.L. (2000). Identification of PKC-isoforms-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* 21, 181–187.
Weatherall, M.W., Telzerow, A.J., Cittadini, E., Kaube, H., Goadsby, P.J. (2010). Intravenous aspirin (lysine acetylsalicylate) in the inpatient management of headache. *Neurology* 75(12), 1098–1103.
Whitten, D.L., Myers, S.P., Hawrelak, J.A., Wohlmuth, H. (2006). The effect of St John's wort extracts on CYP3A: A systematic review of prospective clinical trials. *Br J Clin Pharmacol* 62, 512–526.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time course of the expression and phosphorylation of PKCγ and PKCε by NO donors administration. GTN (A) and SNP (B) increased pPKCγ and pPKCε 1, 2 and 4 h after administration, whereas at 6 h the protein levels returned comparable to control values. Administration of calphostin C (C; 0.2 μg per mouse i.c.v.) counteracted the upregulation of pPKCγ and pPKCε. Increased levels of total PKCγ (C) and PKCε (D) was also detected 2 and 4 h after NO donors' administration.